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Cutting Edge: Evidence for a Signaling Partnership Between Urokinase Receptors (CD87) and L-Selectin (CD62L) in Human Polymorphonuclear Neutrophils[1]

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Cutting Edge: Evidence for a Signaling Partnership Between Urokinase Receptors (CD87) and L-Selectin (CD62L) in Human Polymorphonuclear Neutrophils[1]

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Leukocyte urokinase plasminogen activator receptors (uPARs) cluster at adhesion interfaces and at migratory fronts where they participate in adhesion, chemotaxis, and proteolysis. uPAR aggregation triggers activation signaling even though this glycolipid-anchored protein must associate with membrane-spanning proteins to access the cell interior. This study demonstrates a novel partnership between uPAR and L-selectin in human polymorphonuclear neutrophils. Fluorescence resonance energy transfer demonstrated a direct physical association between uPAR and L-selectin. To examine the role of L-selectin in uPAR-mediated signaling, uPAR was cross-linked and intracellular Ca^{2+} concentrations were measured by spectrofluorometry. A mAb reactive against the carbohydrate binding domain (CBD) of L-selectin substantially inhibited uPAR-mediated Ca^{2+} mobilization, whereas mAbs against the β_2 integrin complement receptor 3 (CR3), another uPAR-binding adhesion protein, had no effect. Similarly, fucoidan, a sulfated polysaccharide that binds to L-selectin CBD, inhibited the Ca^{2+} signal. We conclude that uPAR associates with the CBD region of L-selectin to form a functional signaling complex. ***The Journal of Immunology,*** **2001, 166: 4822–4825.**

Urokinase plasminogen activator receptors (uPAR; CD87)[3] on the plasma membrane exert multiple regulatory effects on leukocyte adhesion, chemotaxis, and activation during recruitment from the circulation to extravascular sites of inflammation. Binding urokinase plasminogen activator (uPA) allows uPAR to concentrate uPA and plasmin activity on the cell surface, a property that may facilitate pericellular proteolysis and cell movement across tissue barriers. uPAR also directly affects leukocyte chemotaxis independently of its associated uPA activity, and uPA-uPAR coupling engages several signal transduction pathways (1–4). One of the distinctive properties of leukocyte uPAR is that it clusters at cell-substratum interfaces and at migratory fronts, prompting us to investigate the possibility that uPAR aggregation can trigger activation signaling (2, 3, 5, 6). We demonstrated that uPAR aggregation induces phosphoinositide hydrolysis and mobilization of intracellular Ca^{2+} in human polymorphonuclear neutrophils (PMN), monocytes, and U937 cells, along with up-regulated PMN degranulation, expression of the β_2 integrin complement receptor 3 (CR3) (CD11b/CD18), and superoxide release (5, 6). In most respects, the results of uPAR aggregation were quite dissimilar to those elicited by binding uPA to uPAR. One of the lingering questions regarding cellular activation through uPAR relates to its linkage to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor (7). Absent any direct connection to the cell interior, uPAR must use more circuitous means to access intracellular signaling. To accomplish this, it forms lateral associations with an array of membrane-spanning proteins, including β_2 integrins (7, 8). Prior work has demonstrated a trimeric signaling complex in PMN comprised of uPA, uPAR, and CR3 (9). Given the distinctions between the effects of uPAR aggregation vs monomeric uPA-uPAR coupling, we sought to determine whether uPAR aggregation involved partner proteins other than β_2 integrins. L-selectin (CD62L) is an adhesion protein that participates in the initial capture of flowing leukocytes and rolling adhesion along the vascular endothelium (10). Its endothelial counterligands principally include carbohydrate structures decorated with sialyl Lewis X, although other ligands have been described (11). We hypothesized that L-selectin would be a candidate uPAR signaling partner because 1) the two proteins aggregate in general proximity to each other during PMN adhesion, 2) L-selectin engagement induces signaling events that resemble those elicited by

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[3] Abbreviations used in this paper: uPAR, urokinase plasminogen activator receptor; uPA, urokinase plasminogen activator; PMN, polymorphonuclear neutrophil(s); CR3, complement receptor 3; CBD, carbohydrate binding domain; TRITC, tetramethylrhodamineisothiocyanate; RET, resonance energy transfer.

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uPAR aggregation, and 3) L-selectin has a carbohydrate binding domain (CBD) that plausibly could interact with heavily glycosylated uPAR (7, 12, 13).

Materials and Methods

Reagents

Purified murine IgG Fc fragments were obtained from Jackson ImmunoResearch (West Grove, PA). Anti-human uPAR mAb (clone 3B10), anti-CD14 mAb (clone 26ic), and integrin mAbs were provided by Robert F. Todd III (University of Michigan Health System, Ann Arbor, MI). Hybridoma cells (3B10) were cultured in vitro, and the IgG2a mAb was purified by protein A-Sepharose (Ab Solutions, Palo Alto, CA). This mAb recognizes an epitope near the uPA binding site, and thus preferentially binds to unoccupied uPAR (6). mAbs were biotinylated with Sulfo-NHS-LC-biotin according to manufacturer's directions (100 μg biotin/mg IgG; Pierce, Rockford, IL). The Dreg-56 hybridoma was obtained from the American Type Culture Collection (Manassas, VA), cultured in vitro (INTEGRA CELLine CL 1000; Integra Biosciences, Ijamsville, MD), and the mAb (IgG1), reactive against the CBD of human L-selectin, was purified with protein A-Sepharose. FITC and tetramethylrhodamineisothiocyanate (TRITC) were obtained from Molecular Probes (Eugene, OR). mAbs were dialyzed (0.15 M carbonate-bicarbonate buffer, pH 9.3, for 16 h at 4°C) and incubated with dyes at a fluorochrome-protein ratio of 40 μg TRITC or 30 μg FITC per mg protein at 25°C for 4 h. The fluorescent conjugates were then separated from unreacted fluorochromes by Sephadex G-25 column chromatography (Sigma, St. Louis, MO). Purified conjugates were dialyzed against PBS at pH 7.4 for 16 h at 4°C.

Purification and stimulation of human PMN

Peripheral blood was obtained from healthy volunteers according to provisions of the University of Michigan Institutional Review Board for Human Subject Research. PMN were isolated to >95% purity by sedimentation in 6% dextran in Ca^{2+}/Mg^{2+}-free PBS, followed by hypotonic lysis of RBCs with H_2O for 30 s and centrifugation through Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, NJ). To cross-link uPAR, cells

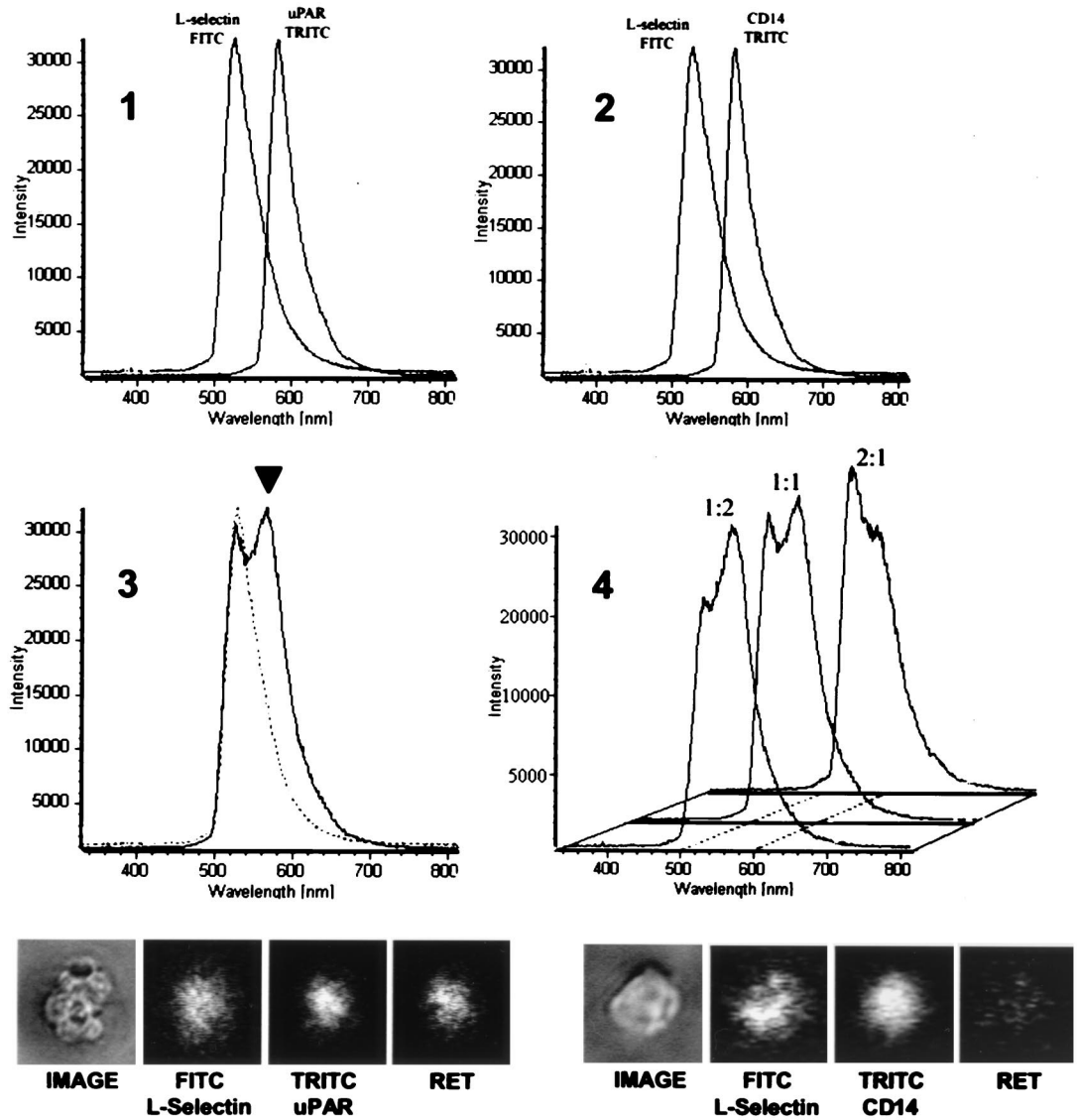

FIGURE 1. Representative emission spectra of labeled PMN. Cells were stained with FITC-conjugated anti-L-selectin (donor) and/or TRITC-conjugated anti-uPAR or TRITC-conjugated anti-CD14 (acceptor), as described in *Materials and Methods*. The emission spectra of cells individually labeled for L-selectin and uPAR are shown in *panel 1*, and the emission spectra of cells individually labeled for L-selectin and CD14 are shown in *panel 2*. *Panel 3* shows substantial RET (▼) between L-selectin and uPAR on a dual-labeled cell (solid line). By contrast, there was no demonstrable RET on a cell dual-labeled for L-selectin and CD14 (dotted line). *Panel 4* shows the dose-response relationship of L-selectin/uPAR RET with a range of donor/acceptor-labeling ratios, as shown. The series of representative fluorescence micrographs also demonstrate the RET between FITC-L-selectin and TRITC-uPAR (*bottom left*), but not between FITC-L-selectin and TRITC-CD14 (*bottom right*).

were suspended in experimental buffer (145 mM NaCl/5 mM KCl/1 mM $MgCl_2$/10 mM glucose/1 mM $CaCl_2$/1% w/v BSA/10 mM HEPES, pH 7.4). Cells were first incubated with murine IgG Fc fragments (150 μg/ml) at 4°C for 15 min to block binding of primary mAbs to Fc receptors. The effectiveness of this blocking step has been demonstrated previously (6). Cells were then incubated with biotinylated anti-uPAR mAb (100 μg/ml) at 4°C for 30 min and washed in experimental buffer. To initiate receptor cross-linking, goat anti-biotin Ab (100 μg/ml; Sigma) was added after warming the cells to 37°C. Previous studies using immunofluorescence microscopy have demonstrated that essentially identical protocols for Ab-mediated uPAR cross-linking in human PMN will produce receptor capping in approximately one-half of labeled cells (9).

Fluorescence microscopy

An axiovert-inverted fluorescence microscope with HBO-100 mercury illumination (Carl Zeiss, New York, NY) interfaced to a Dell 410 workstation via Scion SG-7 video card (Vay Tek, Fairfield, IA) was used. Fluorescence images were collected with an intensified charge-coupled device camera. A narrow bandpass-discriminating filter set was used with excitation at 485DF20 nm and emission of 530DF30 nm for FITC. For rhodamine, an excitation of 540DF20 nm and an emission of 590DF30 nm were used (Omega Optical, Brattleboro, VT). Long-pass dichroic mirrors at 510 and 560 nm were used for FITC and rhodamine, respectively. For energy transfer imaging, the 485DF22, 510LP, and 590DF30 filter combination was used (14).

Single-cell spectra were obtained using a imaging spectrophotometer system (14, 15). Labeled cells were illuminated with an excitation filter at 485DF22 nm and a 510LP dichroic mirror for FITC and resonance energy transfer (RET) experiments. For rhodamine emission spectra, excitation was provided with a 540DF20 nm filter and a 560LP dichroic mirror. The emission spectra were obtained with an Acton-150 (Acton Research, Acton, MA) imaging spectrophotometer fiber-optically coupled to a microscope. The exit port of the spectrophotometer was attached to a Gen-II intensifier coupled with an I-MAX-512 camera (Princeton Instruments, Trenton, NJ). Spectra collection was controlled by a high-speed Princeton ST-133 interface and a Stanford Research Systems DG-535 delay-gate generator (Sunnyvale, CA) and analyzed with Winspec software (Princeton Instruments).

Measurement of intracellular calcium concentration ($[Ca^{2+}]_i$)

Cells were loaded (5×10^6/ml) with the Ca^{2+}-sensitive fluorescent dye Fluo-3/AM (2 μM; Molecular Probes) at 30°C for 30 min in 145 mM NaCl/5 mM KCl/1 mM $MgCl_2$/10 mM glucose/4 mM probenecid/10 mM HEPES, pH 7.4. After pretreatment with Abs as indicated, 2.5×10^6 cells were suspended in 1 ml incubation buffer and prewarmed to 37°C. Fluorescence intensities were then measured with a SLM8000 spectrofluorometer equipped with SLM Spectrum Processor v3.5 software (SLM Instruments, Urbana, IL) using a 1 cm light path cuvette at an excitation wavelength of 505 nm and an emission wavelength of 530 nm. Fluorescence intensities were acquired at 2-s intervals for 300 s with continuous stirring of the cell suspension. These measurements were converted to nanomolar concentrations of $[Ca^{2+}]_i$ by the calibration method of Grynkiewycz et al. (16), using a K_d for Fluo-3 of 864 nM (17).

Statistical analysis

Multiple comparisons were performed with one-way ANOVA with Dunnett's post test for multiple comparisons to a single control. All analyses were performed with GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA).

Results and Discussion

The association of uPAR with L-selectin in human PMN was demonstrated by RET. When cells were illuminated at 485 nm to excite the donor chromophore, robust acceptor emission at 590 nm (evidence of RET) was observed in the presence of both the donor and acceptor (Fig. 1). RET was proportionate to the donor/acceptor-labeling ratio of the cells, and there was no 590 nm emission in the presence of the donor alone. To further demonstrate the specificity of this interaction, no RET was seen when cells were labeled with the combination of a FITC-anti-L-selectin mAb and a TRITC-labeled mAb reactive to another GPI-linked receptor, CD14. Significant RET is indicative of molecular proximity between the fluorochromes within a resolution limit of 7 nm, consistent with a direct "nearest neighbor" interaction between uPAR and L-selectin (18). This type of complex formation is analogous to those described between uPAR and the β_2 integrins CR3 (CD11b/CD18) and CR4 (CD11c/CD18), also demonstrated by RET. In various cell types, uPAR is also found in other assemblies containing, among other proteins, LFA-1, src kinases, β_1 and β_3 integrins, caveolin, mannose-6-phosphate/insulin-like growth factor II-receptor, casein kinase, and nucleolin (7, 8, 19–22).

There are multiple parallels between the signaling events engaged by L-selectin and uPAR, including increased $[Ca^{2+}]_i$, tyrosine phosphorylation, superoxide release, and up-regulated CR3 adhesiveness (7, 12, 13, 23–26). Thus, it is reasonable to suggest that a direct association between these proteins offers the physical basis for a convergence of the two signaling pathways. The next experiments addressed the role of L-selectin in activation signaling through uPAR. uPAR was aggregated and the anti-L-selectin mAb (Dreg 56) was added concurrently to determine its effect on the ensuing Ca^{2+} flux. Fig. 2 shows that the anti-L-selectin mAb suppressed the $[Ca^{2+}]_i$ flux in a dose-related fashion, by as much as 75%. A series of control experiments were also performed to determine whether any anti-CR3 mAbs (some isotype-matched to Dreg 56) had similar effects. Two mAbs against the CR3 α-chain (clones 44 and OKM1), the common β_2 chain (clone TS1/18), and the CR4 α-chain (clone 3.9) did not significantly affect the Ca^{2+} flux induced by uPAR aggregation. The effect of the anti-L-selectin mAb was also specific in that it had no comparable effects (at 50 μg/ml) on Ca^{2+} mobilization induced by the chemotactic peptide FMLP (5×10^{-7} M; 97.9 ± 19.3% control, n = 3). Notably, this signaling pathway is clearly distinct from the one previously defined by binding uPA to uPAR, which induces a Ca^{2+} signal in PMN through an obligate association with CR3 (9). This further emphasizes that uPAR aggregation is a highly distinct signaling mechanism from monomeric occupancy with ligand

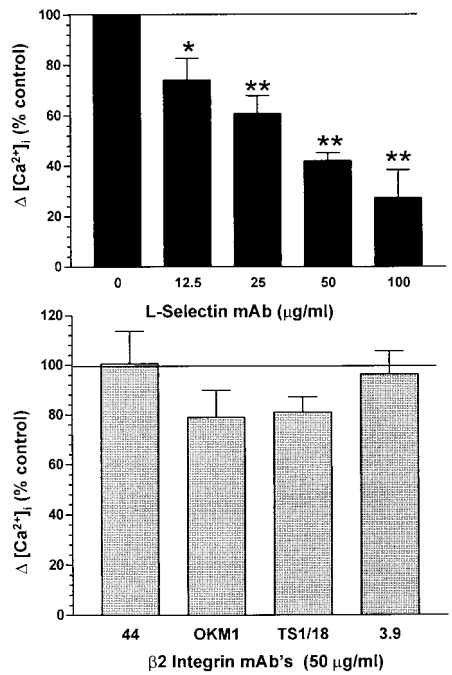

FIGURE 2. *Top*, Addition of anti-L-selectin mAb (Dreg 56) to neutrophils inhibits the increase in $[Ca^{2+}]_i$ induced by uPAR aggregation. Data are expressed as percent control (no mAb), mean ± SEM, $n = 5$. *Bottom*, Identical blocking schemes using mAbs against CR3 α-chain (44, OKM1), or β-chain (TS1/18), and CR4 α-chain (3.9) did not suppress the $[Ca^{2+}]_i$ flux ($n = 3$). Data were analyzed by one-way ANOVA with Dunnett's post test (*, $p < 0.05$; **, $p < 0.01$).

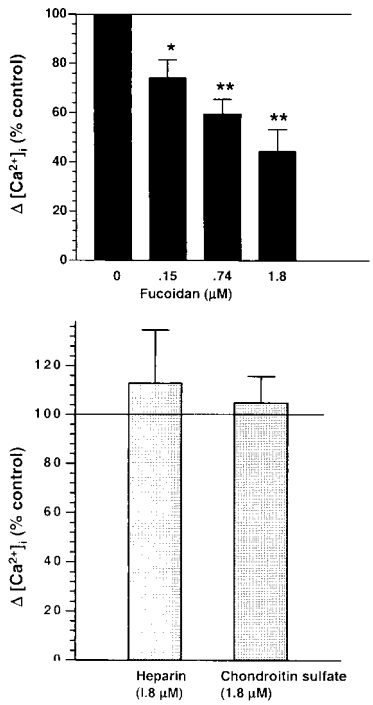

FIGURE 3. *Top*, Fucoidan suppresses the increase in $[Ca^{2+}]_i$ induced by uPAR cross-linking. Data are expressed as percent control (uPAR cross-linking with 0 fucoidan), mean ± SEM, $n = 4$. *Bottom*, Control glycosaminoglycans (heparin, chondroitin sulfate; 1.8 μM) had no significant effect on the change in $[Ca^{2+}]_i$ induced by uPAR cross-linking ($n = 3$). Data were analyzed by one-way ANOVA with Dunnett's post test (*, $p < 0.05$; **, $p < 0.01$).

uPA, both in the pathways engaged downstream and in the composition of the signaling complex. Previously, we demonstrated that uPAR regulates CR3-mediated adhesion to immobilized counterligands, implying that this is due to uPAR binding directly to CR3 (2). Now, further work will be necessary to address the possibility that uPAR also affects CR3 adhesiveness indirectly by regulating CR3 activation through L-selectin engagement (24). It will be important to determine whether there is a dynamic competition between L-selectin and other partner proteins for access to uPAR. Certainly, functioning as a signaling partner for both L-selectin and β_2 integrins broadens the potential influence of uPAR on leukocyte recruitment. It will also be important to know how uPAR forms aggregates. uPAR may draw into clusters by binding directly to its own counterligands (uPA, vitronectin) immobilized on cell surfaces or extracellular matrices. Alternatively, uPAR may be aggregated passively by associating with its partner proteins (L-selectin, integrins) as they form clusters. Recent evidence also indicates that soluble uPAR spontaneously forms dimers and oligomers but it is not known whether this occurs on cell surfaces (27).

Lastly, we sought to determine whether occupancy of L-selectin CBD with a carbohydrate ligand affects the L-selectin/uPAR interaction. Neutrophils were pretreated with fucoidan, a sulfated polysaccharide L-selectin counterligand (25). Fucoidan exerted a significant and dose-related suppression of uPAR-mediated Ca^{2+} mobilization, whereas equimolar concentrations of control glycosaminoglycans with similar charge density (heparin, chondroitin sulfate) had no effect (Fig. 3). These findings suggest that uPAR interacts with the CBD of L-selectin, and that, in effect, uPAR may compete with L-selectin ligands to form a competent signaling assembly. In such a scheme, uPAR aggregation may only initiate activation signaling if it is in proximity to L-selectin that is not actively engaged in adhesion to carbohydrate ligands.

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